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(54) Title: PEROXIDASE-CATALYSED FLUORESCENCE

(57) Abstract

An assay method comprises bringing together a leuco-dye and an oxidant and a peroxidase which catalyses oxidation of the leuco-dye to a fluorescent dye. The assay is performed in the presence of an enhancer which is a substituted aromatic hydroxy or amine or borate compound which enhances fluorescent light output. The dye may be for example a fluorescein or rhodamine or oxazine dye. Preferred enhancers include 6-hydroxybenzothiazole and 6-hydroxy-2-naphthoic acid.

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PEROXIDASE-CATALYSED FLUORESCENCE

This invention concerns an assay system involving the well known reaction between a peroxidase enzyme, e.g. horseradish peroxidase (HRP), an oxidant e.g. hydrogen peroxide, and a substrate or leuco-dye which on catalysed oxidation is converted to a product observable by its colour or because it has chemiluminescent or fluorescent properties. In an assay format, one of the three reagents is present in unknown concentration and is or represents an analyte. The reagents may be present in solution or any one of the three reagents may be immobilised e.g. on a membrane.

In one such system, the substrate is luminol or a related compound which on enzyme catalysed oxidation generates light by chemiluminescence. A p-substituted phenol may be present to enhance the chemiluminescence. The use of such phenols in this assay system has been described in the following patents: EP 87959; EP 116454; EP 296752; USP 4,729,950; USP 5,106,732; USP 5,279,940.

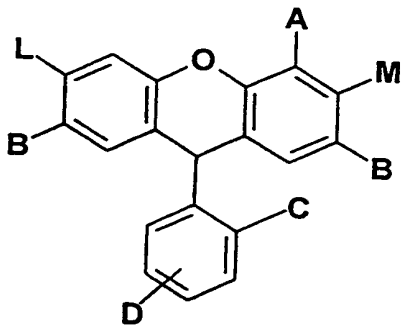
In the peroxidase enzyme assay system, other known substrates are leuco-dyes which are oxidised to fluorescent dyes. This invention results from the discovery that, with certain families of such leuco-dyes, phenols and certain other aromatic compounds can be used to enhance the fluorescent light output. This enhancement may be by increasing the signal, or by reducing background light, so as to increase a signal-noise ratio, or by means of both effects together. As shown in the examples below, different families of dyes behave differently in this respect.

Thus the invention provides an assay method which comprises bringing together a leuco-dye and an oxidant and a peroxidase

which catalyses oxidation of the leuco-dye to a fluorescent dye, wherein the assay is performed in the presence of an enhancer which is a substituted aromatic hydroxy or amine or borate compound which enhances fluorescent light output.

Assay systems in which a fluorescent signal is detected are quicker and cheaper to perform than those in which the signal is radioactive; and more accurate and sensitive than those in which the signal is colorimetric and provide greater signal intensity than chemiluminescence; and offer the possibility of multiplexing by using several different dyes simultaneously. Enzyme-catalysed fluorescence has the advantage over conventional fluorescent detection with single fluors in that it allows amplification of a fluorescent signal due to enzyme turnover. The assay systems of this invention thus have the potential to compete with known assay systems using other signals.

The leuco-dye may be a xanthane-based compound, including the families of dihydrofluoresceins and dihydrorhodamines, having the following structures:



Where L and M are the same or different and each is independently selected from $-OR^1$ or $-NR^2R^3$, where R^1 is H or $-COR^4$, R^4 is a C1 - 12 aryl or alkyl group added to increase the stability of the leuco dye and prevent non-specific oxidation to the fluorescent dye, preferably $-CH_3$,

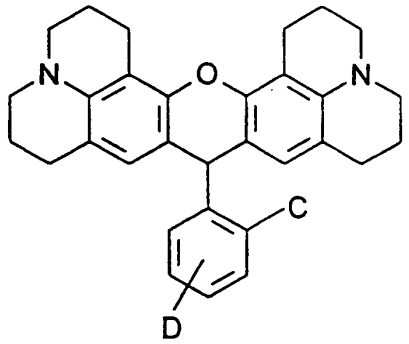
R^2 and R^3 are the same or different and each is independently selected from H, or any lower alkyl group of C1-10 which may carry a carboxyl or a sulphonic acid group, preferably CH_3, or C_2H_5,

A is H or any lower alkyl group of C1-10, preferably CH_3,

B is any electron withdrawing or donating group, preferably H, C1-C12 alkyl or alkoxy, CN, $(CH_2)_2CO_2H$, or a halogen (I, Br, Cl, F),

C and D are the same or different and each is independently selected from H, COOH, $COOCH_3$ or SO_3H, provided that when C is COOH this may form a lactone with position 9,

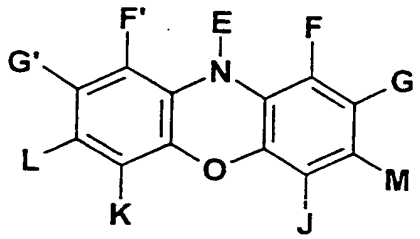
or when L and/or M = $-NR^2R^3$, a fused ring structure may be formed between A and/or B as shown below:



The following dyes are preferred:

- 2',7'-dichlorodihydrofluorescein diacetate (2',7'-dichlorofluorescin diacetate).
- 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (5-(and-6)-carboxy-2',7'-dichlorofluorescin diacetate).
- dihydrofluorescein diacetate.
- dihydrorhodamine 123.
- dihydrorhodamine 6G.

Alternatively the leuco-dye may be an oxazine-based compound including the following structures:



Where L and M are the same or different and each is independently selected from $-OR^1$ or $-NR^2R^3$, where R^1 = H or $-COR^4$,

R^4 is a C1 - 12 aryl or alkyl group added to increase the stability of the leuco dye and prevent non-specific oxidation to the fluorescent dye, preferably $-CH_3$,

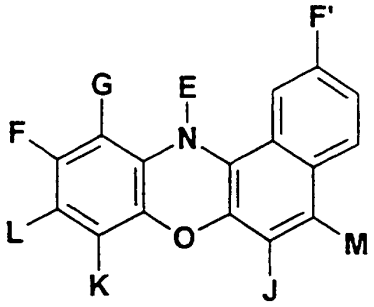
R^2 and R^3 are the same or different and each is independently selected from H, or any lower alkyl group of C1-10 which may carry a carboxyl or a sulphonic acid group, preferably CH_3, or C_2H_5,

F, G, J, K, F' and G' are the same or different and each is independently selected from H, or any lower alkyl or alkoxy group of C1-10 or aryl group which may carry a carbonyl or sulphonic acid group, preferably CH_3, C_2H_5, $(CH_2)CO_2H$, or a halogen (I, Br, Cl, F), or CN,

Or F-G and/or F'-G' form a ring structure which may or may not be conjugated to the chromophore,

E is a leaving group which may include a function that modifies the physical properties of the dye such as solubility, preferably $-COR^5Z^1$ where R^5 is C1-C12 alkyl and Z^1 is H or carboxyl or sulphonic acid.

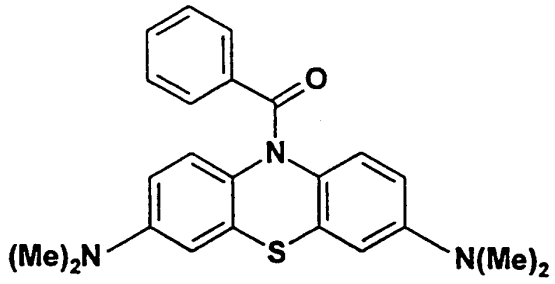
Phenoxazine based compounds may include the following structures (where F-G forms a ring structure):



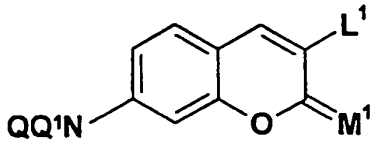
Where J, K, L, M, E, F, F' and G are as defined above.

Also envisaged are thiazine and phenothiazine dyes, having structures as above except that the ring O atom is replaced by S, e.g. methylene blue. Other dyes envisaged include coumarin and extended coumarin dyes including disperse red, squarate dyes and cyanine dyes. In their fluorescent or oxidised state, all these dyes have a conjugated unsaturated structure that is not present in their reduced or leuco-dye state.

Preferred is the dye N-benzoyl-leuco-methylene blue.



Also envisaged are leuco-derivatives of extended coumarins based upon the following structure:

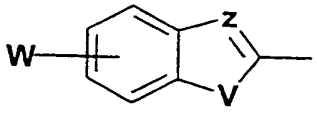


Where Q and Q^1 are the same or different and each is independently selected from H, or any lower alkyl group of C1-10 which

may carry a carboxyl or sulphonic acid group, preferably C_2H_5 or $(CH_2)_2COOH$.

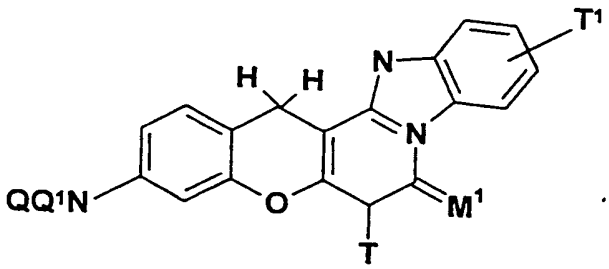
M^1 is selected from O or NH.

L^1 is a benzimidazole, benzoxazole or benzothiazole ring system, with or without additional substituents. For example:



Where Z=N, V=NH, N-alkyl, O or S and W is any substituent.

An example of an extended leuco-coumarin structure is given below:



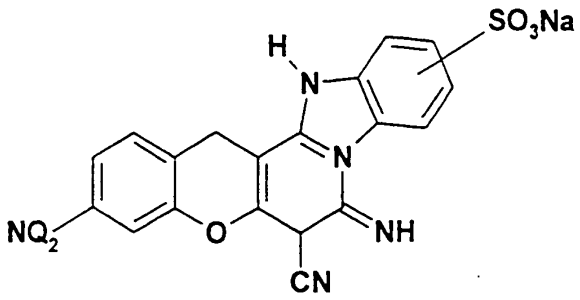
Where Q and Q^1 are as given above.

M^1 is selected from O, NH or NCOAr.

T^1 substituents in the benzimidazole ring may be H or any common substituent, but preferably $-SO_3H$ to enhance water solubility.

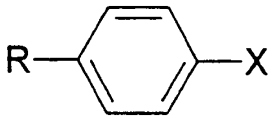
T is preferably CN or $CONH_2$.

The preferred dyes in this family are Disperse Red 277 leucodye I (N-ethyl derivative) and II (N-butyl derivative).



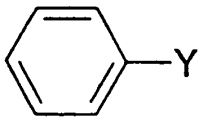
(I) Q = Et
(II) Q = n-Butyl

The enhancer may be *p*-substituted phenol or amine or boronic acid of the formula



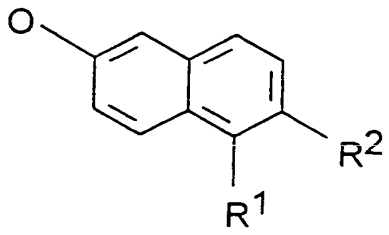
where R = -OH, or $-NH_2$ or $-B(OH)_2$

X = Halogen (I, Cl, Br); amine; sulphonic acid; or a salt or ester thereof; any group that extends the resonance, preferably imidazole, -CH=CH-COOH, carboxy, nitrile, formyl, or $-(CH=CH)_n$-Ar (where n is 0-4), or N=N-Ar, where Ar has the formula:



where Y is hydrogen, hydroxy, nitrile, amine, sulphonic acid, formyl or a salt or ester thereof. Phenol compounds are described in EP 116 454. Organic amine compounds are described in USP 4,729,950. Boronic acid phenol compounds are described in US Patent 5, 512,451.

Or the enhancer may be substituted naphthol of the formula



where R^1 and R^2 are the same or different and each is independently selected from hydrogen, carboxylic acid, nitrile, amine, sulphonic acid, formyl, Cl, Br or I; or a salt or ester thereof; or any other group that extends the resonance of the structure. Or R^1 and R^2 together form a ring which extends conjugation of the naphthyl system and is preferably an imidazole or oxazole or thiazole.

The nature of the peroxidase enzyme is not material to the invention. Conveniently HRP maybe used. Typical concentrations are in the range 0.001 pM to 1 nM.

The nature of the oxidant is not material to the invention. Preferred are per-compounds such as sodium perborate and hydrogen peroxide. Typical concentrations are in the range 10 μM to 300 mM particularly 50 μM to 10 mM.

The leuco-dye is preferably used at a concentration in the range 0.5 μM to 200 mM particularly 1 μM to 1 mM. The enhancer is preferably used at a concentration in the range 1 μM to 100 mM particularly 10 μM to 10 mM. The assay may suitably be performed at a temperature in the range 10 - 50°C in a buffered aqueous medium at pH in the range 6 - 10 particularly 7 - 9. It will often be convenient to perform multiple assays in parallel in individual wells of a Multiwell plate.

In one assay format, all the agents are present in solution. Any one reagent may be present in unknown concentration and may be or represent an analyte. A preferred formulation in solution is 0.01mM N-

acetyl-3,7-dihydroxyphenoxazine, 0.5mM sodium perborate, 0.1M 6-hydroxybenzothiazole, in 0.1M Tris pH 8.0.

In another assay format, an analyte may be immobilised, either on the surface of an assay vessel or as a blot on a porous membrane. It is preferred that a blot be blocked by application of an aqueous fluid containing casein or milk powder; and that casein or milk powder be also included in the liquid phase components of the assay; this has the effect of improving the signal-to-noise ratio. It is often convenient to immobilise the peroxidase enzyme on a membrane blot, and to measure the concentration of the peroxidase enzyme as an assay for the immobilised analyte. A preferred formulation for membrane blots is 0.01mM dihydrorhodamine-123, 0.1mM 6-hydroxy-2-naphthoic acid, 0.5mM sodium perborate, 0.01% casein in 0.1M borate buffer pH 9.5. Dihydrorhodamine 123 has the advantage that it does not migrate readily on the membrane.

Equipment for observing fluorescent signals is readily available, and the wavelength is readily adjusted to detect output from the particular fluorescent dye being used. The assay may be performed on a qualitative or quantitative basis. When the assay is quantitative, the fluorescent light output may be measured at a particular time interval (after excitation) or may be the sum of all fluorescent light output over a defined period of time.

The following examples illustrate the invention. Examples 1 to 3 show the use of rhodamine dyes. Examples 4 to 6 show the use of fluorescein dyes. Examples 7 to 9 show the use of phenoxazine dyes. These families of dyes respond differently to enhancers:

- The rhodamine family shows a large enhancement of signal together with (generally) a small increase in background.
- The fluorescein family shows a moderate enhancement of signal - the effect on background is not clear.

- The phenoxazine family shows a small enhancement of signal but with (generally) a slightly decreased background giving rise to an improved signal:noise ratio.

EXAMPLES

Example 1. Enhancement of fluorescence from Dihydrorhodamine in solution.

20µl of a 1nM solution of horseradish peroxidase (HRP) in 0.1M borate buffer pH 8.5 was added to 80µl of detection reagent containing dihydrorhodamine 123 (final concentration 0.1mM), sodium perborate (final concentration 1mM) and enhancer compounds tested at final concentrations of 3.0, 1.0, 0.3, 0., 0.03 and 0mM in borate buffer pH 8.5. The enhancers were prepared as 5mM and 15mM stocks in the same buffer. The borate buffer was prepared from orthoboric acid and NaOH.

The reactions were carried out in clear, flat-bottomed 96-well microtitre plates. They were imaged using the Vistra FluorImager SI with an excitation wavelength of 488nm (from an Argon laser), a 570nm emission filter and with the PMT set to 500V.

Unlike enhancement of chemiluminescence with luminol, the enhancers do not generally appear to act by reducing the background signal

For results see Table 1.

Table 1. Relative enhancement activity of phenols with dihydrorhodamine in solution.

Enhancer	Max. enhancement[1] (*mM Enhancer*)	Max. Signal: Noise ratio[2] (*mM Enhancer*)	Effect on background[3]
PIMP	270 (*1.0*)	67.5 (*0.3*)	x5[4]
BiPCN	149 (*0.3*)	123.6 (*0.3*)	x5
PIP	148 (*1.0*)	88 (*3.0*)	<0.5[5]
BiPCA	159 (*1.0*)	79 (*0.3*)	<0.5
PHCA	121 (*0.3*)	129 (*0.3*)	Decreases[6]
HBT	286 (*1.0*)	124 (*1.0*)	<0.5
NA	262 (*0.3*)	111 (*0.3*)	<0.5
BrN	114 (*0.1*)	82 (*0.3*)	<0.5
AP	50 (*0.3*)	71 (*1.0*)	Decreases
MA	31 (*0.3*)	54 (*0.3*)	Decreases
UNENHANCED	0	2.2	-

1. The maximum enhancement values were obtained by comparing the maximum fluorescent signal for each enhancer relative to the signal from the system without enhancer at the same time point. Values in parentheses show the concentration of enhancer.

2. The signal:noise ratios (S/N) were obtained by comparing the fluorescent signals from the enhanced reactions in the presence and absence of HRP at the optimum time point for each enhancer (t=20mins for MA, t=10mins for all the rest) .

3. Effect on background as the enhancer concentration increases.

4. Background increases as enhancer concentration increases to a maximum of approximately 5 x unenhanced value.

5. Background increases as enhancer concentration increases, but to less than half of the unenhanced value.

6. Enhancers cause a decrease in fluorescent background (but by no greater than half of the unenhanced value).

PIMP	: 4-Imidazol-yl-phenol
BiPCN	: 4'-Hydroxy-4'-biphenyl carbonitrile
PIP	: 4-Iodophenol
BiPCA	: 4-(4'-hydroxyphenyl)benzoic acid
PHCA	: 4-Hydroxycinnamic acid
HBT	: 6-Hydroxybenzothiazole
NA	: 6-Hydroxy-2-naphthoic acid
BrN	: 6-Bromo-2-naphthol
AP	: 4-Acetamidophenol
MA	: Methoxyaniline

Example 2. Relative activity of enhancer phenols with dihydrorhodamine on membrane blots.

A series of dot blots of mouse immunoglobulin G (IgG) were prepared on PVDF membrane at the following concentrations: 50ng, 5ng, 500pg, 50pg, 10pg, 5pg, 1pg, 0.5pg and 0pg.

Before each experiment the blots were wetted in methanol and rinsed in water before blocking in 5% milk powder in phosphate-buffered saline (pH 7.5) containing 0.1% (v/v) Tween-20 (PBS-T). The blocking step took place overnight at 2-4°C.

The blots were next washed in PBS-T for 1x15 min and 2x5min before incubating with biotinylated anti-mouse IgG diluted 1:5000 in PBS-T for 1 hour at room temperature. After washing as before, the

blots were incubated with streptavidin-HRP conjugate at a dilution of 1:5000 in PBS-T for 1 hour at room temperature. The blots were washed again in PBS-T for 1x15 min and 4x5 min before the addition of detection reagent.

The detection reagent contained 0.1mM dihydrorhodamine-123, 0.5mM sodium perborate and enhancer compounds at 1mM, 0.5mm and 0.1mM (except PIP which was tested at 10mM, 2mM and 1mM) in 0.1M borate buffer pH 9.5.

The blots were imaged using the Vistra FluorImager SI with an excitation wavelength of 488nm (from an Argon laser), a 570nm emission filter and with the PMT set to 500V. For results see Table 2.

Table 2.

Enhancer	Limit of detection, pg IgG (*mM Enhancer*)	Max. Signal: Noise ratio (*at t hours*)
BiPCA	1.0 (*0.5mM*)	40.3 (*2.7*)
BiPCN	5.0 (*0.5mM*)	39.0 (*1.3*)
NA	5.0 (*0.1mM*)	37.3 (*1.3*)
βN	5.0 (*0.5mM*)	34.3 (*1.3*)
PPP	5.0 (*0.1mM*)	26.1 (*1.3*)
PIP	10.0 (*2mM*)	20.3 (*2.7*)

The maximum signal: noise ratio for each enhancer was obtained from the 5ng dot in comparison to the negative control on each blot.

βN : β-naphthol

PPP : p-phenylphenol

Example 3. Detection of Southern blots with the preferred formulation of dihydrorhodamine-123.

Hind III-treated human genomic DNA was separated by agarose gel electrophoresis at loadings of 2μg-0.5μg and Southern blotted onto Hybond N+ membrane. Fluorescein-labelled N-Ras probe was prepared by the random prime reaction described in the Amersham random prime labelling and detection kit booklet (RPN 3040) using a 1-3 hour incubation. This was hybridised to the genomic blots at a probe concentration of 10-12.6 ng/ml for up to 16 hours at 60°C. The blots were washed at 60°C in an excess of 1xSSC, 0.1%SDS(w/v) for 15 minutes, then in 0.5xSSC, 0.1% SDS for a further 15 minutes.

The blots were rinsed in TBS (10mM Tris-HCl, 300mM NaCl, pH 7.5), then blocked for 1 hour at room temperature in an excess of liquid block diluted 1:10 in TBS.

Sheep anti-fluorescein HRP conjugate was added at a 1:1000 dilution in 0.5% BSA (w/v) in TBS for 1 hour at room temperature. The blots were next washed in an excess of 0.1% Tween-20 in TBS for 3x 10 minutes before the addition of detection reagent. The detection reagent contained 0.01mM dihydrorhodamine-123, 0.1mM 6-hydroxy-2-naphthoic acid, 0.5mM sodium perborate, 0.01% casein in 0.1M borate buffer pH 9.5. The blots were imaged using the Vistra FluorImager SI with an excitation wavelength of 488nm (from an Argon laser), a 570nm emission filter and with the PMT set to 500V. The limit of detection was 0.25 pg DNA.

Example 4. Enhancement of fluorescence from dichlorofluorescin diacetate in solution.

20µl of a 2.5nM solution of horseradish peroxidase (HRP) in 0.1M borate buffer pH 8.5 was added to 80µl of detection reagent containing 2',7'-dichlorofluorescin diacetate (DCF diacetate) (final concentration 0.1mM), sodium perborate (final concentration 0.25mM) and enhancer compounds tested at final concentrations of 10.0, 4.0, 1.2, and 0mM in borate buffer pH 8.5. The enhancers were prepared as 10mM stocks in the same buffer. The borate buffer was prepared from orthoboric acid and NaOH.

The reactions were carried out in clear, flat-bottomed 96-well microtitre plates. They were imaged using the Vistra FluorImager SI with an excitation wavelength of 488nm (from an Argon laser), a 530nm emission filter and with the PMT set to 450V.

The results shown in Table 3 below are taken at t=16 minutes after the start of the reaction.

Table 3.

Enhancer	Max. enhancement (*mM Enhancer*)	Max. Signal: Noise ratio (*mM Enhancer*)
PIP	206 (*4.0*)	43.5 (*4.0*)
BiPCA	103 (*1.2*)	126 (*1.2*)
UNENHANCED	-	1.09

Example 5. Relative activity of enhancer phenols with dichlorofluorescin diacetate on membrane blots.

λ-Hind III DNA was labelled with fluorescein in a random prime reaction as described in Example 3. This labelled DNA was then dotted onto Hybond N+ membrane at loadings of 3pg, 1pg, 0.3pg, 0.1pg, 0.03pg and 0pg. The blots were blocked in a mixture of a 1;20 dilution of liquid block and 0.5% BSA in TBS pH 7.5 (see Example 3) for 1 hour at room temperature. The blots were now treated as described in Example 3, except that the final washes were 2x10 min and 2x 5 min in TBS-Tween. The detection reagent contained 0.02mM 2',7'-dichlorofluorescin diacetate, 0.5mM sodium perborate and a range of enhancers at 2.0, 1.0, 0.5 and 0.1mM in 0.1M borate buffer pH 9.5. The blots were imaged using the Vistra FluorImager SI with an excitation wavelength of 488nm (from an Argon laser), a 530nm emission filter and with the PMT set to 450V. For results see Table 4.

Table 4.

Enhancer	Limit of detection, pg DNA	Signal: Noise ratio
BiPCA	0.1	3.5
BiPCN	0.1	9.8
PPP	0.1	4.1
NA	0.1	3.5
PHCA	0.1	1.8
βN	0.3	3.3
MA	0.3	4
PIP	0.3	1
HHBP	1.0	2.5
BrN	1.0	1.8
AP	1.0	1.6

HHBP : 4-Hydroxy-3-[3-(*p*-hydroxyphenyl)-1-oxo-2-propenyl]-2H-1-benzopyran-2-one.

Example 6. Relative activity of enhancer phenols with fluorescein derivatives on membrane blots.

Hybond N+ blots were prepared and treated as described in Example 5. The detection reagents contained 0.5mM sodium perborate; 0.01mM substrate consisting of either 2',7'-dichlorofluorescin diacetate, dihydrofluorescein diacetate or 5-(&-6-)-carboxy-2',7'-dichlorofluorescin diacetate. The enhancers tested were 1mM NA, 0.5mM BiPCA and 0.5mM PHCBA in 0.1M borate buffer pH 9.5. The blots were imaged using the

Vistra FluorImager SI with an excitation wavelength of 488nm (from an Argon laser), a 530nm emission filter and with the PMT set to 500V.

For results see Table 5.

Table 5.

Enhancer	2',7'-DCF diacetate		5-(&-6-)-carboxy-2',7'-DCF diacetate		dihydrofluorescein diacetate	
	pg DNA[1]	S/N ratio[2]	pg DNA[1]	S/N ratio[2]	pg DNA[1]	S/N ratio[2]
BiPCA	0.03	5.5	0.03	4.9	0.1	4.9
NA	0.1	3.5	0.1	2.8	0.1	4.5
PHCBA[3]	0.03	3.0	0.1	3.5	0.1	1.5

1. Limit of detection on dot blot.
2. Maximum signal:noise ratio for the 3pg DNA dot.
3. PHCBA: trans-4-(3-propenoic acid) phenyl boronic acid.

Example 7: Effect of enhancers on the fluorescent signal from N-acetyl-3,7-dihydroxyphenoxazine.

20µl of a 0.5nM solution of horseradish peroxidase (HRP) in 0.1M Tris buffer pH 7.5 was added to 80µl of detection reagent containing N-acetyl-3,7-dihydroxyphenoxazine (final concentration 0.01mM in 100µl), sodium perborate (final concentration 0.5mM in 100µl) and enhancer compounds tested at final concentrations of 3.0, 1.0, 0.3, 0.1, 0.03 and 0mM in 0.1M Tris buffer pH 7.5. The enhancers were prepared as 10mM stocks in the same buffer.

The reactions were carried out in clear, flat-bottomed 96-well microtitre plates. They were imaged using the Vistra FluorImager SI with

an excitation wavelength of 488nm (from an Argon laser), a 590nm emission filter and with the PMT set to 500V.

For results see Table 6.

Table 6

Enhancer	Max. enhancement[1] (*mM Enhancer*)	Max. Signal: Noise ratio[2] (*mM Enhancer*)	Enhanced background as % of unenhanced background[3]
NA	2.17 (*0.03*)	864 (*0.03*)	31
HBT	3.7 (*0.1*)	598 (*0.1*)	49
PHCA	2.0 (*0.03*)	655 (*0.03*)	31
AP	0.56 (*0.03*)	661 (*0.03*)	10
MA	0.49 (*0.01*)	378 (*0.03*)	36
βN	1.18 (*0.01*)	354 (*0.01*)	51
BiPCN	3.8 (*0.1*)	331 (*0.1*)	91
BiPCA	3.2 (*0.1*)	311 (*0.03*)	80
PIP	3.5 (*0.1*)	240 (*0.1*)	116
PIMP	3.2 (*0.1*)	161 (*0.03*)	170
UNENHANCED	-	197	100

1. The maximum enhancement values were obtained at the initial time point (t = 3 minutes) by comparing the maximum fluorescent signal for each enhancer relative to the signal from the system without enhancer. Values in parentheses show the concentration of enhancer.

2. The signal:noise ratios (S/N) were obtained by comparing the fluorescent signals from the enhanced reactions in the presence and absence of HRP.

3. Enhanced background as % of unenhanced background at t=3 mins. Although some enhancers increase the background above that of the unenhanced reaction, the majority of enhancers have the effect of lowering the background. The increase in background over time is also lower in the presence of many enhancers so that the optimal signal:noise ratio is between 30-120 minutes.

Example 8. HRP dilution curve with N-acetyl-3,7-dihydroxyphenoxazine in solution with three different enhancers.

20µl of a range of HRP dilutions was added to 80µl detection reagent. The final concentrations of the reactants in 100µl was as follows: 100pM to 0.01pM HRP; 0.01mM N-acetyl-3,7-dihydroxyphenoxazine; 0.5mM sodium perborate; 0.1mM 6-hydroxybenzothiazole or 0.1mM 4-iodophenol or 0.03mM 6-hydroxy-2-naphthoic acid in 0.1M Tris buffer pH 7.5. The unenhanced reaction was carried out in 0.1M sodium phosphate buffer, pH 7.0, since it had previously been established that this was the optimum pH for this reaction.

The reactions were carried out in clear, flat-bottomed 96-well microtitre plates. They were imaged using the Vistra FluorImager SI with an excitation wavelength of 488nm (from an Argon laser), a 590nm emission filter and with the PMT set to 500V.

The readings were taken 2 hours after the start of the reaction. The limits of detection (set at 2x background) were 0.11pmolL^{-1} for HBT, 0.12pmolL^{-1} for NA, 0.16pmolL^{-1} for PIP and 0.18pmolL^{-1} for the unenhanced reaction.

Example 9. Comparison between N-acetyl-3,7-dihydroxyphenoxazine and TMB in the detection of human tumour necrosis factor-α.

The Biotrak™ human tumour necrosis factor-α [(h)TNFα] ELISA kit from Amersham International was used as a test system in both its normal (RPN 2758) and high sensitivity (RPN 2788) forms. The assays were carried out according to the protocols in the kit booklets. The chromogenic substrate, 3,3',5,5'-tetramethylbenzidine (TMB) was incubated with a (h)TNFα standard curve for 30 minutes as detailed in the protocol booklet before stopping with sulphuric acid and immediately reading the absorbance at 405nm.

The fluorogenic reaction mixture contained 0.01mM N-acetyl-3,7-dihydroxyphenoxazine (Phx) ; 0.5mM sodium perborate; and 0.1mM 6-hydroxybenzothiazole in 0.1M Tris buffer pH 7.5.

The reactions were carried out in the antibody coated-clear, flat-bottomed 96-well microtitre plates that were part of the kit.
The TMB absorbance readings were carried out in a Labsystems Multiskan MCC/340 plate reader at room temperature and a Molecular Dynamic's BioLumin 960 instrument at 37°C.

The fluorescent readings were taken using the Vistra FluorImager SI with an excitation wavelength of 488nm (from an Argon laser), a 590nm emission filter and with the PMT set to 500V at room temperature, and a Molecular Dynamic's BioLumin 960 instrument with an excitation filter of 560nm and an emission filter of 595nm at 37°C.

N-Acetyl-3,7-dihydroxyphenoxazine was approximately four-fold more sensitive than TMB irrespective of assay system or instrument used.

See Table 7 for results.

Table 7

Substrate	Detection method	Limit of detection (pg/ml)[1]	ELISA system sensitivity	Instrument
TMB	Colour	21.5	Normal	Multiskan
Phx	Fluorescence	6.3	Normal	FluorImager SI
TMB	Colour	0.39	High sensitivity	BioLumin 960
Phx	Fluorescence	0.1	High sensitivity	BioLumin 960

1. Limit of detection of (h)TNFα from a standard curve, with a limit of detection set at 2x background.

Example 10. Comparison of enhanced fluorescence from dihydrorhodamine and N-acetyl-3,7-dihydroxyphenoxazine against enhanced luminol chemiluminescence in solution.

DHR-123: 20µl of a 1nM solution of horseradish peroxidase (HRP) in 0.1M borate buffer pH 8.5 was added to 80µl of detection reagent containing dihydrorhodamine 123 (final concentration 0.1mM), sodium perborate (final concentration 1mM) and enhancer compounds tested at final concentrations of 3.0, 1.0, 0.3, 0., 0.03 and 0mM in borate buffer pH 8.5. The enhancers were prepared as 5mM and 15mM stocks in the same buffer. The borate buffer was prepared from orthoboric acid and NaOH. Each assay was performed in duplicate.

N-AcPhx: 20µl of a 0.5nM solution of horseradish peroxidase (HRP) in 0.1M Tris buffer pH 7.5 was added to 80µl of detection reagent containing N-acetyl-3,7-dihydroxyphenoxazine (final concentration 0.01mM in 100µl), sodium perborate (final concentration 0.5mM in 100µl) and enhancer compounds tested at final concentrations of 3.0, 1.0, 0.3, 0.1, 0.03 and 0mM in 0.1M Tris buffer pH 7.5. The enhancers were prepared as 10mM stocks in the same buffer. Each assay was performed in duplicate. The reactions were carried out in clear, flat-bottomed 96-well microtitre plates. They were imaged using the Vistra FluorImager SI with an excitation wavelength of 488nm (from an Argon laser), a 570nm emission filter (for DHR-123) or a 590nm emission filter (for N-AcPhx)and with the PMT set to 500V.

Luminol: 10µl of a 5nM solution of HRP was added to 90µl detection reagent containing 3mM sodium perborate, 0.5mM luminol and enhancer compounds at final concentrations of 1.0, 0.5, 0.1, 0.01mM and 0mM in 0.1M borate buffer pH 8.5. The borate buffer was prepared as above. The reactions were carried out in Microfluor "B" black, flat-bottom, 96-well microtitre plates from Dynatech Laboratories Inc. (US). The plates were read in an Amerlite plate reader from Amersham International plc (UK) set at a dwell time of 0.2 seconds/well. Each assay was performed in duplicate.

It can be seen that the enhancer compounds have different effects upon the three substrates so that the best enhancer for one substrate is not necessarily the best enhancer for the other two. Unlike enhancement of chemiluminescence with luminol and fluorescence with N-acetyl-3,7-dihydroxyphenoxazine, the enhancers do not generally appear to act by reducing the background signal with dihydrorhodamine 123.

For results see Table 8.

Table 8. Relative enhancement activity of phenols with dihydrorhodamine, N-acetyl-3,7-dihydroxyphenoxazine and luminol in solution.

Enhancer	Max. enhancement[1]		
	DHR-123	N-AcPhx	Luminol[2]
PIMP	270	3.2	2606.6
BiPCN	149	3.8	1690.5
PIP	148	3.5	1510.1
BiPCA	159	3.2	941.1
PHCA	121	2.0	214.2
HBT	286	3.7	105.9
NA	262	2.17	18.5
BrN	114	nd	nd
AP	50	0.56	112.3
MA	31	0.49	nd
Effect on background	Increases with increased enhancer concentration.	Decreases slightly with increased enhancer concentration.	Decreases with increased enhancer concentration.

1. The maximum enhancement values were obtained by comparing the maximum fluorescent signal for each enhancer relative to the signal from the system without enhancer at the same time point.

2. The maximum enhancement values were obtained by comparing the maximum fluorescent signal for each enhancer relative to the mean signal from the system without enhancer at a single time point.

Example 11. Comparison between N-acetyl-3,7-dihydroxyphenoxazine, dihydrorhodamine 123, 2',7'-dichlorodihydrofluorescein diacetate and 5'-(and- 6)-carboxy-2', 7'-dichlorodihydrofluorescein diacetate in the detection of human tumour necrosis factor-α.

The Biotrak™ human tumour necrosis factor-α [(h)TNFα] ELISA kit from Amersham International was used as a test system in its normal sensitivity form (RPN 2758) . The assays were carried out according to the protocols in the kit booklets.

The chromogenic substrate, 3,3',5,5'-tetramethylbenzidine (TMB) was incubated with a (h)TNFα standard curve for 30 minutes as detailed in the protocol booklet before stopping with sulphuric acid and immediately reading the absorbance at 405nm.

The fluorogenic reaction mixture contained either10μM N-acetyl-3,7-dihydroxyphenoxazine (Phx) or 0.25μM dihydrorhodamine (DHR)123, 2',7'-dichlorodihydrofluorescein (DCF) diacetate or 5'-(and- 6)-carboxy-2', 7'-dichlorodihydrofluorescein diacetate plus 0.5mM sodium perborate; and either no enhancer or 0.1mM 6-hydroxybenzothiazole (HBT) or 0.03mM 6-hydroxy-2-naphthoic acid (NA) in 0.1M Tris buffer pH 7.5.

The reactions were carried out in the antibody coated-clear, flat-bottomed 96-well microtitre plates that were part of the kit.

The TMB absorbance readings were carried out in a Molecular Dynamic's BioLumin 960 instrument at 37°C set to absorbance mode.

The fluorescent readings were taken using the BioLumin 960 instrument from Molecular Dynamics with the appropriate excitation and emission filters for each substrate at 37°C (see Table 9 for details).

The signal:noise levels were determined for each of the four substrates over a range of (h)TNFα concentrations over time. Table 9 below gives the results for 1ng/ml (h)TNFα at t=20 minutes.

Table 9

	Filter sets	SIGNAL: NOISE RATIOS		
	Ex/Em	UNENHANCED	NA	HBT
N-AcPhx	560/595	49.61	77.41	97.67
DHR 123	505/535	1.16	3.61	3.12
2',7'-DCF diacetate	505/535	1.15	1.93	1.55
5-(&-6-)-carboxy-2',7'-DCF diacetate	505/535	0	1.78	1.53
TMB	Absorbance	20.01	nd	nd

Example 12. Effect of enhancer on Disperse Red leucodyes

The absorbance spectra of Disperse Red 277 (imine form) leucodye I (N-ethyl derivative) and II (N-butyl derivative) were investigated in the presence and absence of horseradish peroxidase (HRP) and enhancer phenol 4-(4'-hydroxyphenyl)benzoic acid (BiPCA). The reaction mixture was comprised of the following reagents at the final concentrations given below:

0.1 mM Disperse Red 277 leucodye I or II, 3 mM sodium perborate, 0.5 mM BiPCA, 500 pM HRP in 0.1 M borate buffer (prepared from orthoboric acid and sodium hydroxide) pH 8.5 or 0.1 M phosphate buffer pH 6.0.

The reactions were carried out in a quartz cuvette and the changes in absorbance spectra monitored in a Phillips PU 8720 UV/Visible spectrophotometer over the range 250-700 nm. The fluorescent spectra were monitored in a Hitachi F-4500 fluorimeter at PMT=700V, and slit widths of Ex/Em 2.5 nm/5 nm.

For results see Table 10.

Table 10

	Absorbance		Fluorescence	
	λmax/nm	A (ODU)[1]	λem/nm[2]	Comments
DR(I)	563	0.196	573[3]	Leucodye
DR(I)	564	0.109	Nd	+ Perborate
DR(I)	564	0.147	Nd	Perborate, BiPCA
DR(I)	554 (523)[4]	0.4	573[3]	HRP, BiPCA, Perborate
DR(II)	567	0.094	578[5]	Leucodye
DR(II)	567	0.098	Nd	+ Perborate
DR(II)	566	0.109	578[5]	Perborate, BiPCA
DR(II)	567	0.142	578[5]	+ HRP
DR(II)	558 (526)[4]	0.67	578[5]	HRP, BiPCA, Perborate

nd: Not determined.

1. Absorbance in optical density units

2. Emission wavelength. In phosphate buffer pH 6 there is a red shift of approximately 10 nm in both the excitation and emission wavelengths.

3. Values determined from emission scan with excitation wavelength of 555 nm.

4. Excitation at 523 nm (DRI) and 526 nm (DRII) will not give rise to fluorescence and are probably due to dye aggregation.

5. Values determined from emission scan with excitation wavelength of 559 nm.

Example 13. Enhancement of HRP-catalysed N-benzoyl-leuco-methylene blue

The fluorescent signal from N-benzoyl-leuco-methylene blue was investigated in the presence and absence of the enhancer phenol 4-(4'-hydroxyphenyl)benzoic acid (BiPCA).

A substrate mix was prepared as follows: 8.0 ml 0.1 M borate buffer pH 8.5, 4.0 ml 1-methyl-2-pyrrolidinone, 0.466 ml 60 mM sodium perborate, 0.075 ml 100 mM N-benzoyl-leuco-methylene blue in dimethylformamide (DMF). To 100 µl of this mixture was added 50 µl of BiPCA in DMF over the concentration range 2-20 mM (final concentration). Finally, 1.25 units of HRP in 5 µl was added to the reaction mixture which was left for ten minutes before imaging.

The reactions were carried out in clear, flat-bottomed 96-well microtitre plates. They were imaged using a prototype Vistra FluorImager 575 with a helium/neon laser exciting at 633 nm and a 670 nm emission filter.

For results see table 11.

Table 11

BiPCA [mM]	Enhancement[1]	Signal:Background ratio[2]	Increase in background[3]
0	-	8.2	-
2	4.2	19.3	1.8
4	15.2	52.3	2.4
8	37.8	31.9	9.7
16	82.6	114.5	5.9
20	77.7	126.7	5.0

1. Signal from enhanced reaction relative to signal from unenhanced reaction.

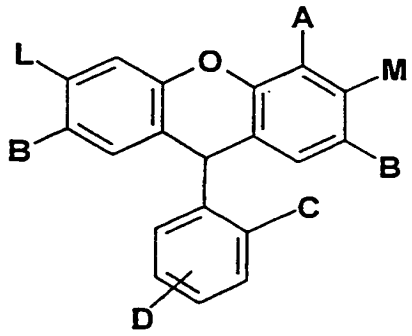
2. The signal:background ratios were obtained by comparing the signals in the presence and absence of HRP.

3. The rise in background with enhancer concentration was determined relative to the background signal from the unenhanced reaction.

CLAIMS

1. An assay method which comprises bringing together a leuco-dye and an oxidant and a peroxidase which catalyses oxidation of the leuco-dye to a fluorescent dye, wherein the assay is performed in the presence of an enhancer which is a substituted aromatic hydroxy or amine or borate compound which enhances fluorescent light output.

2. An assay method as claimed in claim 1, wherein the leuco-dye is a xanthene-based compound having the following structure



Where L and M are the same or different and each is independently selected from $-OR^1$ or $-NR^2R^3$, where R^1 is H or $-COR^4$,

R^4 is a C1 - 12 aryl or alkyl group added to increase the stability of the leuco dye and prevent non-specific oxidation to the fluorescent dye, preferably $-CH_3$,

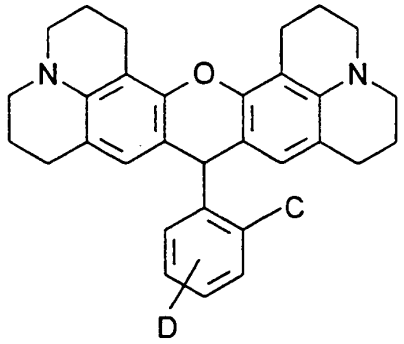
R^2 and R^3 are the same or different and each is independently selected from H, or any lower alkyl group of C1-10 which may carry a carboxyl or a sulphonic acid group, preferably CH_3, or C_2H_5,

A is H or any lower alkyl group of C1-10, preferably CH_3,

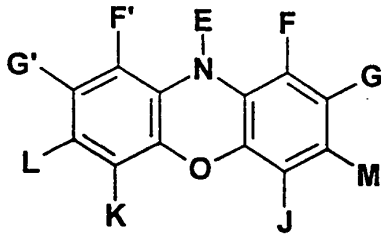
B is any electron withdrawing or donating group, preferably H, C1-C12 alkyl or alkoxy, CN, $(CH_2)_2CO_2H$, or a halogen (I, Br, Cl, F),

C and D are the same or different and each is independently selected from H, COOH, $COOCH_3$ or SO_3H, provided that when C is COOH this may form a lactone with position 9,

or when L and/or M = $-NR^2R^3$, a fused ring structure may be formed between A and/or B as shown below:



3. An assay method as claimed in claim 1, wherein the leuco-dye is an oxazine-based compound having the following structure:



Where L and M are the same or different and each is independently selected from $-OR^1$ or $-NR^2R^3$, where R^1 = H or $-COR^4$,

R^4 is a C1 - 12 aryl or alkyl group added to increase the stability of the leuco dye and prevent non-specific oxidation to the fluorescent dye, preferably $-CH_3$,

R^2 and R^3 are the same or different and each is independently selected from H, or any lower alkyl group of C1-10 which may carry a carboxyl or a sulphonic acid group, preferably CH_3, or C_2H_5,

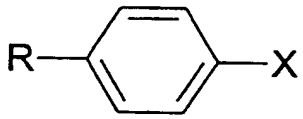
F, G, J, K, F' and G' are the same or different and each is independently selected from H, or any lower alkyl group of C1-10 or aryl group, preferably CH_3, C_2H_5, $(CH_2)CO_2H$, or a halogen (I, Br, Cl, F),

Or F-G and/or F'-G' form a ring structure which may or may not be conjugated to the chromophore,

E is a leaving group which may include a function that modifies the physical properties of the dye such as solubility, preferably $-COR^5Z^1$ where R^5 is C1-C12 alkyl and Z^1 is H or carboxyl or sulphonic acid.

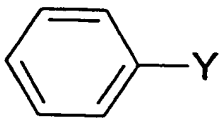
4. An assay method as claimed in claim 1, wherein the leuco-dye is a phenoxazine-based compound, or a thiazine, a phenothiazine, methylene blue, a coumarin, an extended coumarin, e.g. disperse red, a squarate or a cyanine dye.

5. An assay method as claimed in any one of claims 1 to 4, wherein the enhancer is a *p*-substituted phenol or amine or boronic acid of the formula



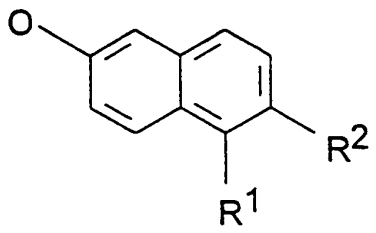
where R = -OH, or $-NH_2$ or $-B(OR)_2$,

X = Halogen (I, Cl, Br); amine; sulphonic acid; or a salt or ester thereof; any group that extends the resonance, preferably imidazole, -CH=CH-COOH, carboxy, nitrile, formyl, or $-(CH=CH)_n$-Ar (where n is 0-4), or N=N-Ar, where Ar has the formula:



where Y is hydrogen, hydroxy, nitrile, amine, sulphonic acid, formyl or a salt or ester thereof.

6. An assay method as claimed in any one of claims 1 to 4, wherein the enhancer is a substituted naphthol of the formula



where R^1 and R^2 are the same or different and each is independently selected from hydrogen, carboxylic acid, nitrile, amine, sulphonic acid, formyl, Cl, Br or I or a salt or ester thereof; or any other group that extends the resonance of the structure. Or R^1 and R^2 together form a ring which extends conjugation of the naphthyl system and is preferably an imidazole or oxazole or thiazole.

7. An assay method as claimed in any one of claims 1 to 6, wherein all the reagents are in solution.

8. An assay method as claimed in claim 7, wherein the leuco-dye is N-acetyl-3,7-dihydroxyphenoxazine and the enhancer is 6-hydroxybenzothiazole.

9. An assay method as claimed in any one of claims 1 to 6, wherein the peroxidase is immobilised on a membrane.

10. An assay method as claimed in claim 9, wherein the leuco-dye is dihydrorhodamine 123 and the enhancer is 6-hydroxy-2-naphthoic acid.

11. An assay kit comprising:

- a supply of a leuco-dye selected from dihydrofluorosceins, dihydrorhodamines and oxazines,
- a supply of an oxidant,
- a supply of a peroxidase enzyme,
- and a supply of an aromatic hydroxy or amine or borate compound as an enhancer.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 98/01888

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/58 C12Q1/28

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 252 747 A (EASTMAN KODAK) 13 January 1988 see claims ---	1,5
X Y	EP 0 103 784 A (ENZYME TECHNOLOGY COMPANY) 28 March 1984 see claim 1 ---	1 2-11
Y	EP 0 630 884 A (LUMIGEN INC) 28 December 1994 see page 3, line 39 - line 48 ---	2-11
Y	EP 0 116 454 A (SECR SOCIAL SERVICE BRIT) 22 August 1984 cited in the application see the whole document --- -/--	1,5,6

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of theinternational search	Date of mailing of the international search report
14 October 1998	26/10/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Fletcher, A

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INTERN IONAL SEARCH REPORT

International Application No
PCT/[redacted]98/01888

C.(Continuation) DOCUMENTS CONSID[redacted]TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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